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**14. ABSTRACT**

The long-term goal of our research project is to develop a novel therapy for HER-2/neu overexpressing prostate cancer. Previous studies have shown that the HER-2/neu homodimer constitutively activates the PI(3)K-Akt-NF-κB signal transduction pathway. During the research period, we examined whether the PI(3)K-Akt-NF-κB signal transduction pathways are involved in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. As a first step we investigated whether modulation of the PI3K-Akt - NF-κB signals affects TRAIL-induced cytotoxicity. We observed that acetyl salicylic acid (ASA: aspirin), amiloride, and quercetin inhibit the PI(3)K-Akt signal transduction pathway and promote TRAIL-induced cytotoxicity in HER-2/neu overexpressing human prostate cancer cells. The second step we examined how these chemical compounds enhances TRAIL cytotoxicity. We observed that ASA promotes TRAIL cytotoxicity by down-regulating SURVIVIN gene expression. We believe that the outcome of these studies provides information to support the development and clinical application of TRAIL for the treatment of HER-2/neu overexpressing human prostate cancer.

**15. SUBJECT TERMS**
HER-2/neu; TRAIL; Amiloride; Aspirin; Quercetin; PI(3)K; Akt; NF-κB; Survivin

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## Table of Contents

## Introduction

It is believed that, as in other neoplastic diseases, accumulation of genetic alterations causes and promotes prostate cancer even though the exact molecular mechanisms underlying the onset and progression of prostate cancer are unknown. In the United States, prostate cancer is the leading cancer diagnosis and the second leading cause of cancer-related deaths in men (Jemal et al, 2007). The incidence of prostate cancer for men under the age of 40 years is 1 in 10,000 compared with 1 in 7 for those aged over 60 (Jemal et al, 2007). In as many as 10% to 50% of men with prostate cancer the disease will progress from androgen-dependent to androgen-independent growth (Isaacs, 1994; Goktas, 1999) and spread to the pelvic lymph nodes and bone. This development of an androgen-independent phenotype leads to the incurable hormone-refractory state of the disease, suggesting the need for better treatment strategies. Novel agents such as nucleotide-based targeted therapies, small-molecule inhibitors, antiangiogenic agents, novel cytotoxic therapeutics, and calcitriol have been proposed (Hadaschik et al, 2007). Although there are a substantial number of novel protocols including hormonal therapy as well as conventional chemo- and immunotherapy, only limited treatment options are available for prostate cancer because chemotherapy and radiotherapy have been found to be largely ineffective, and metastatic disease frequently develops even after surgery (Petrylak, 1999; Pisters, 1999; Richie, 1999). Death is the result of metastatic hormone refractory disease in virtually the majority of patients. Hence, the development of a novel and effective therapeutic strategy to effectively inhibit hormone refractory prostate cancer is urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is possibly one of the best candidates for a new form of cytokine therapy. TRAIL, a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family, induces apoptosis in a broad range of cancer cells types but spares normal cells and tissues (Ashkenazi and Dixit, 1999; Ashkenazi et al,1999). Preclinical studies clearly demonstrate that TRAIL has excellent antitumoral activity (Ashkenazi et al, 1999; de Jong et al, 2001; Chawla-Sarkar et al, 2003). However, many tumor cells have been shown to be resistant to TRAIL (Bouralexis et al, 2003, Tillman et al, 2003). Several researchers have reported that TRAIL resistance can be overcome by various sensitizing agents such as chemotherapeutic drugs (Lee et al, 2001; Fulda et al, 2004), cytokines (Park et al, 2002), and matrix metalloprotease inhibitors (Nyormoi et al, 2003) that are able to render TRAIL-resistant tumor cells sensitive to TRAIL.

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (aspirin, ASA), have been used as chemopreventive agents of cancers to induce apoptosis or to reduce the incidence of tumor formations in a variety of organs, i.e., colon (Qiao et al, 1998), lung (Hosomi et al, 2000), and stomach (Wong et al, 1998). ASA is known to act by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), the rate limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. ASA was also shown to be effective in the inhibition of ultraviolet radiation and carcinogen-induced tumor formation in animal models (Bair et al, 2002; Wargovich et al, 2000). In this study, we examined whether ASA in combination with TRAIL increases TRAIL-induced apoptotic death in AIPC (androgen independent prostate cancer, which is also referred to as hormone-refractory prostate cancer). For this study, we employed human prostate adenocarcinoma LNCaP cell line and its derivatives (C4, C4-2, and C4-2B) (Thalmann et al, 1994). This is an excellent model system which will help improve our understanding of the mechanisms of androgen-independence and osseous metastasis, and tumor-host determinants of PSA expression (Thalmann et al, 1994; Wu et al, 1994; Thalmann et al, 2000). Our studies demonstrate that ASA augments TRAIL-induced apoptosis by down-regulating survivin gene expression and by decreasing binding affinity of the transcription factor E2F-1 to survivin promoter, which results in decrease in the intracellular level of survivin.

**Body**

The long-term goal of our research project is to develop a novel therapy for hormone-refractory prostate cancer. Hormone-refractory prostate cancer cells that resist conventional chemotherapeutic agents can be induced to undergo apoptotic death by engagement of death receptors expressed on their membrane surface by TRAIL. In this budget period, we examined whether aspirin (ASA) promotes TRAIL cytotoxicity by down-regulating survivin gene expression. As androgen-dependent LNCaP and its derivative androgen-independent C4, C4-2B, and C4-2B cells were treated with ASA, we observed that TRAIL-induced cytotoxicity was promoted. Our observations are illustrated below:

***ASA promotes TRAIL-induced apoptotic cell death***

To examine the effect of ASA on TRAIL-induced apoptotic death in human prostatic adenocarcinoma LNCaP cells and their derivatives C4, C4-2, and C4-2B cells, cells were pretreated with ASA and treated with TRAIL in the presence of ASA. Figs. 1A and 1B show that little or no cytotoxicity was observed with 5 mM ASA alone or 200 ng/ml TRAIL alone. Similar results were observed with DNA fragmentation assay (Fig. 1C). Additional studies also show that pretreatment with ASA followed by treatment with TRAIL caused PARP cleavage, the hallmark feature of apoptosis, in LNCaP and its derivatives (Fig. 1D). With the pretreatment of ASA (0.5-5 mM), TRAIL-induced apoptosis was promoted in an ASA concentration dependent manner in LNCaP and its derivatives (Fig. 1E). These data demonstrate that TRAIL-induced apoptotic cell death was promoted by pretreatment with ASA regardless of androgen dependency, since LNCaP cells are androgen-dependent while C4, C4-2 and C4-2B cells are androgen-independent.

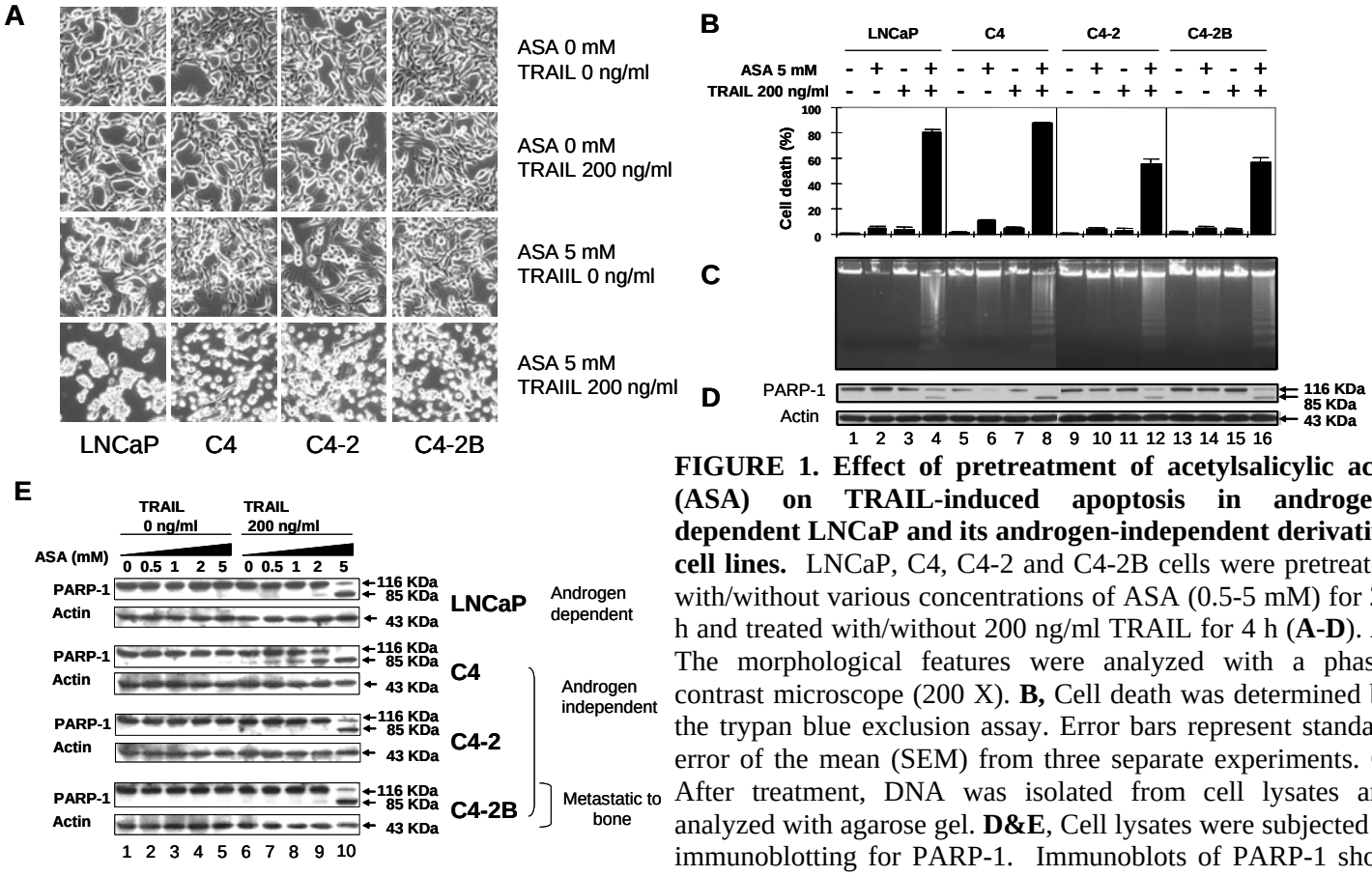


**FIGURE 1. Effect of pretreatment of acetylsalicylic acid (ASA) on TRAIL-induced apoptosis in androgen-dependent LNCaP and its androgen-independent derivative cell lines.** LNCaP, C4, C4-2 and C4-2B cells were pretreated with/without various concentrations of ASA (0.5-5 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h (**A-D**). **A**, The morphological features were analyzed with a phase-contrast microscope (200 X). **B,** Cell death was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **C**, After treatment, DNA was isolated from cell lysates and analyzed with agarose gel. **D&E**, Cell lysates were subjected to immunoblotting for PARP-1. Immunoblots of PARP-1 show the 116-kDa PARP-1 and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

### *Caspase activation is responsible for ASA-pretreated TRAIL-induced apoptosis*

Additional experiments were conducted to investigate whether pretreatment with ASA followed by treatment with TRAIL activates caspases. Western blot analysis shows that procaspase-8 (55 kDa) was cleaved to the intermediates (41 and 43 kDa) by pretreatment with ASA and treatment with TRAIL in androgen-dependent LNCaP and androgen-independent and metastatic LNCaP subline C4-2B cells. The combined treatment of TRAIL and ASA also resulted in an increase in caspase-9 activation as well as caspase-3 activation in a dose dependent manner in both LNCaP and C4-2B cells (Fig. 2). The precursor form of caspase-9 and -3 was cleaved to the active form of 37 and 17 kDa, respectively. As expected by Fig. 1, ASA alone did not activate caspases. Fig. 2 shows that PARP (116 kDa) was cleaved yielding a characteristic 85-kDa fragment in the presence of 200 ng/ml TRAIL and ASA (2–5 mM) in both LNCaP and C4-2B cells. The cleavage of PARP was not observed by treatment with ASA alone.

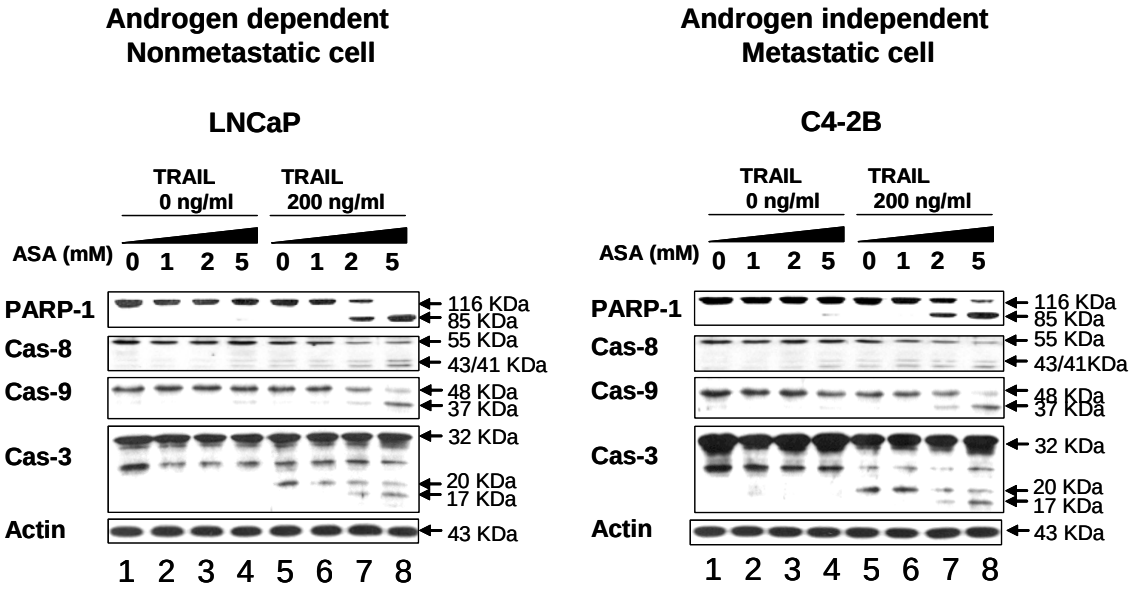


**FIGURE 2. Effect of pretreatment of ASA on TRAIL-induced proteolytic cleavage of PARP-1 and activation of caspases in LNCaP and C4-2B cells.** Cells were pretreated with various concentrations of ASA (1-5 mM) for 20 h and treated with/without TRAIL (200 ng/m) for 4 h, and then harvested. Equal amounts of protein (20 μg) for cell lysates were separated by SDS-PAGE and subjected to immunoblotting for PARP-1, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa) and cleaved intermediates (41 and 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa) and cleaved active form (17 kDa). Immunoblots of PARP-1 show the 116-kDa PARP and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

### *ASA downregulates the intracellular level of survivin*

We previously reported that ASA does not significantly alter the total cellular levels of the TRAIL receptors (DR4, DR5, and DcR2) and anti-apoptotic proteins (FLIPL, FLIPS, IAP-1, IAP-2, and Bcl-xL) (Kim et al, 2005). In this study, we observed that ASA treatment resulted in a significant decrease in the level of survivin among IAP family proteins in LNCaP, C4, C4-2, and C4-2B cells (Fig. 3A). The reduction of survivin during treatment with ASA (5 mM ) in LNCaP and its derivative prostate cancer cells were dependent upon exposure time (Fig. 3B). The role of survivin in TRAIL sensitivity was examined by knockdowning survivin gene expression. Data from Figure 4 show that the expression of survivin was effectively inhibited by survivin siRNA transfection in androgen-dependent LNCaP and androgen-independent and bone metastatic C4-2B cells. TRAIL-induced PARP-1 cleavage was also observed in both siRNA transfected cell lines (Fig. 4). These results suggest that downregulation of survivin leads to an increase in sensitivity to TRAIL regardless of androgen dependency.

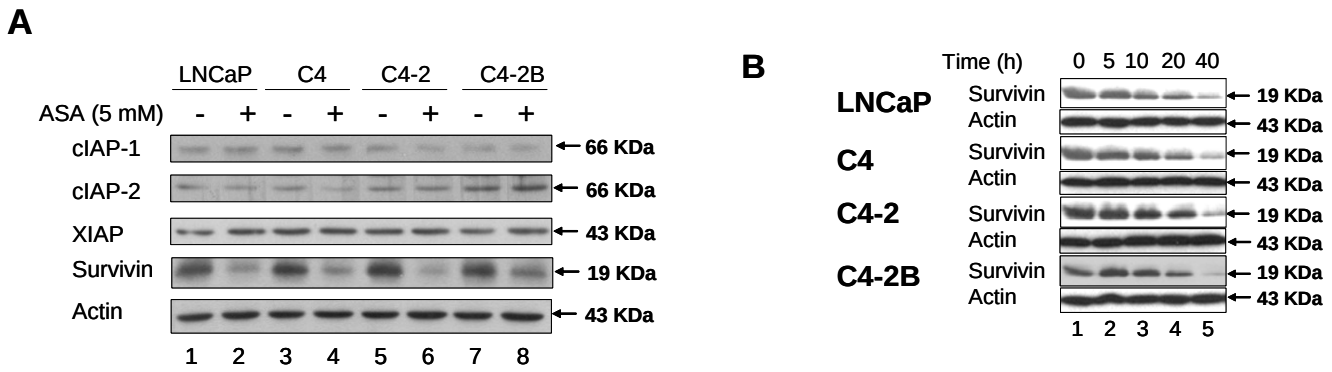


**FIGURE 3. ASA-mediated downregulation of survivin expression in LNCaP, C4, C4-2 and C4-2B cells. A**, Cells were treated with/without 5 mM ASA. Cells were harvested 20 hr after treatment and subjected to western blot. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-cIAP-1, anti-cIAP-2, anti-XIAP, anti-survivin or anti-actin antibody. Actin was shown as an internal standard. **B**, Time course expression of survivin. Cells were treated with 5 mM ASA for various times (5-40 h) and harvested. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-survivin or anti-actin antibody.

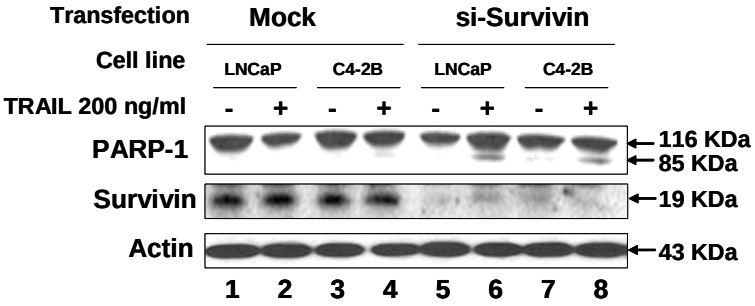


**FIGURE 4. Role of survivin in TRAIL-induced apoptosis in LNCaP and C4-2B cells.** Cells were transfected with survivin siRNA or control siRNA and incubated for 36 h. Cells were then treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP-1, anti-survivin, or anti-actin antibody.

**ASA-suppressed *SURVIVIN* gene transcription is mediated through inhibiting DNA binding activity of E2F-1**

We further examined whether downregulation of *SURVIVIN* gene expression by treatment with ASA is due to inhibition of transcriptional activity. Data from RT-PCR in Fig. 5A show that the level of *SURVIVIN* mRNA was significantly decreased during treatment with 5 mM ASA. These results suggest that the reduction of survivin levels during treatment with ASA was related to suppression of *SURVIVIN* gene transcription. To confirm our observations, pLuc and pLuc-1430 (the 1430 base of *SURVIVIN* promoter sequence followed by pLuc) plasmids were transfected to LNCaP cells and luciferase activities were determined with or without 5 mM ASA. Figure 5B clearly shows that luciferase activity from cell lysate of pLuc-1430 transfected cells was dramatically decreased in the presence of ASA. These results suggest that downregulation of *SURVIVIN* gene expression by treatment with ASA is mediated through inhibition of transcriptional activity. It is well known that survivin promoter region contains binding sites of several putative transcription factors such as Sp1, Stat-3, E2F-1, p53, etc. (Fig. 6A). We hypothesized that ASA affects binding affinity of these transcription factors in the *SURVIVIN* promoter region and subsequently inhibits transcription of the *SURVIVIN* gene. To test the hypothesis, we chose two potential candidates, Sp1 and E2F-1, because there are 8 putative binding sites for these transcription factors in the *SURVIVIN* promoter region. Data from EMSA assay show that Sp1 from the nuclear extracts of LNCaP and C4-2B bound to the Sp1 specific oligomer. This binding activity was not affected by treatment with ASA. Unlike the DNA binding activity of Sp1, that of E2F-1 was changed in the presence of ASA (Fig. 6D). Even though the intracellular level of E2F-1 was not significantly altered in the presence of ASA (Fig. 6C), data from ChiP assay clearly demonstrate that ASA treatment markedly decreased recruitment of E2F-1 to the proximal site of survivin promoter (Fig. 6D) in both cell lines. To investigate whether ASA specifically alters E2F-1 binding activity to the *SURVIVIN* promoter region, total E2F-1 binding activity to sonicated chromatin was determined by immunoprecipitation with anti-E2F-1 antibody followed by immunoblotting with anti-histone H3. Figure 6E shows that total E2F-1 binding activity wasn't significantly changed in the presence of ASA. Taken together, our data suggest that specific disruption of E2F-1-binding activity to survivin promoter occurs by treatment with ASA. The role of E2F-1 in the downregulation of

*SURVIVIN* gene expression was confirmed by knockdowning E2F-1. Figure 6F shows that transfection with E2F-1 siRNA effectively decreased the intracellular level of E2F-1 and significantly suppressed the level of survivin in LNCaP and C4-2B cells.

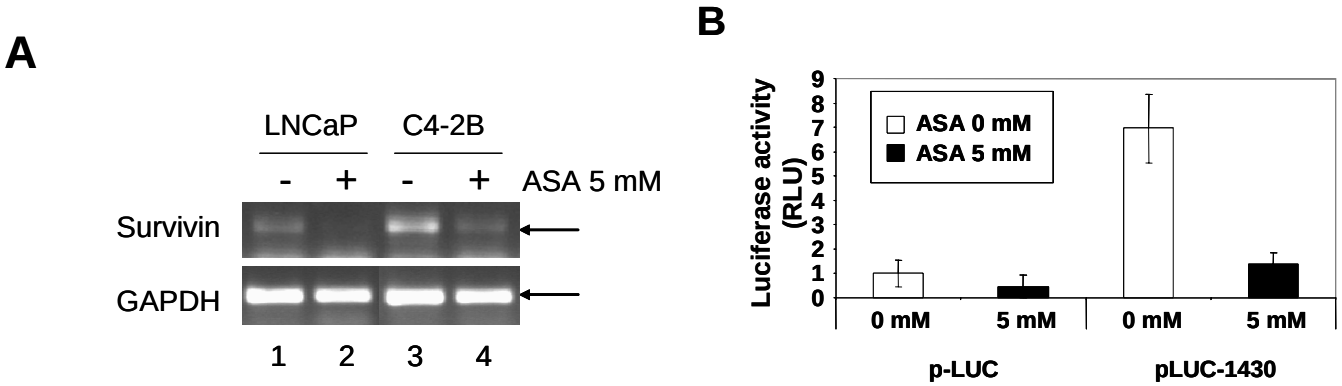


**FIGURE 5. Transcriptional downregulation of *SURVIVIN* gene expression by treatment with ASA. A**, RT-PCR analysis was performed for detecting *SURVIVIN* or *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)* expression in LNCaP and C4-2B cells. *GAPDH* is shown as an internal standard. **B**, LNCaP or C4-2B cells were transfected with pLuc or p1430Luc plasmids and incubated for 30 h. After incubation, cells were treated with/without 5 mM ASA for 20 h. Cells were lysed and luciferase activities were measured with a luminometer. Error bars represent standard error of the mean (SEM) from three separate experiments.

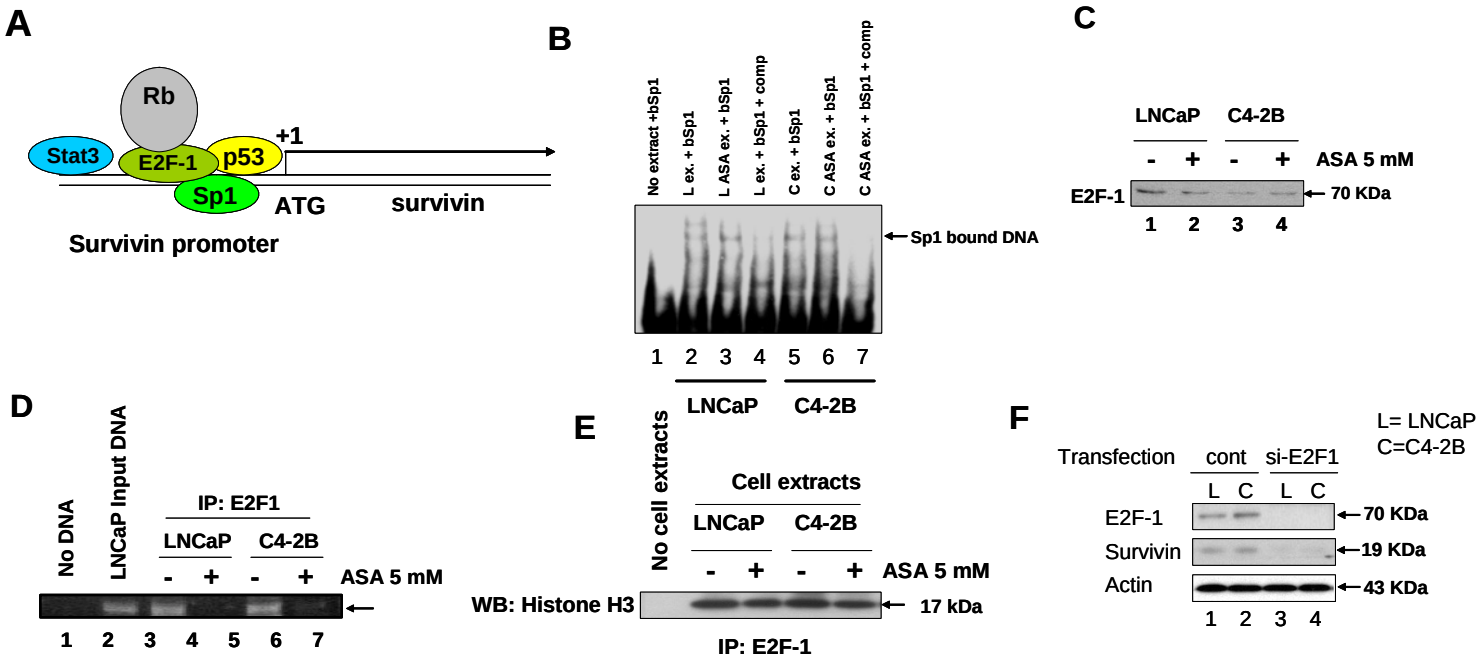


**FIGURE 6. Transcription factor binding activity in *SURVIVIN* promoter. A**, Diagram of putative transcription factors in the *SURVIVIN* promoter. *SURVIVIN* promoter contains stat-3 binding sites (-1231~ -1009, 3 putative binding sites), E2F-1 (-43~-38) and Sp1 binding sites (-1409 ~ -24, 8 putative binding sites). **B-E**, LNCaP and C4-2B cells were treated with 5 mM ASA for 20 h. **B**, Their nuclear extracts were incubated with biotin-labeled *SURVIVIN* promoter oligonucleotide at room temperature for 30 min. Gel mobility shift assays for Sp1 binding activity were performed as described under the "Experimental Procedures." **C**, Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-E2F-1 antibody. **D**, Cells were sonicated and chromatin fragments were immunoprecipitated with anti-E2F-1. The binding of E2F-1 on *SURVIVIN* promoter was analyzed by PCR. **E**, Chromatin fragments were immunoprecipitated with anti-E2F-1 antibody. Interaction beteen E2F-1 and histone H3 was performed with anti-histone H3 antibody. **F**, LNCaP and C4-2B cells were transfected with E2F-1 siRNA or control siRNA and incubated for 48 h. Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-E2F-1, anti-survivin, or anti-actin antibody.

**Key research accomplishments:**

We previously reported that ASA enhances TRAIL-induced cytotoxicity. In this study we observed that ASA promotes TRAIL-induced apoptosis in androgen-dependent LNCaP cells as well as its derivative androgen-independent C4, C4-2, and C4-2B cells. The mechanism of this enhancement is likely shown to be due to downregulation of *SURVIVIN* gene expression. These results suggest that ASA in combination with TRAIL can be an effective therapeutic strategy against hormone refractory prostate cancer.

**Reportable Outcomes**

1. Yoo J, Lee YJ. (2007) Effect of hyperthermia and chemotherapeutic agents on TRAIL-induced cell death in human colon cancer cells. J. Cell Biochem., In press.

2. Yoo J, Lee YJ. (2007) Effect of hyperthermia on TRAIL-induced apoptotic death in human colon cancer cells: development of a novel strategy for regional therapy. J Cell Biochem., 101:619-630.

3. Song JJ, An JY, Kwon YT, Lee YJ. (2007) Evidence for two modes of development of acquired tumor necrosis factor-related apoptosis-inducing ligand resistance. Involvement of Bcl-xL. J Biol Chem., 282:319-328.

4. Kim YH, Lee YJ. (2007) TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. J Cell Biochem., 100:998-1009.

5. Yoo J, Kim HR, Lee YJ. (2006) Hyperthermia enhances tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human cancer cells. Int J Hyperthermia, 22:713-728.

**Conclusions**

We have shown that ASA can potentiate TRAIL-induced apoptotic death in androgen-dependent LNCaP cells and their derivative androgen-independent C4, C4-2, and C4-2B cells. Our studies suggested that ASA-promoted TRAIL cytotoxicity is mediated through down-regulating survivin and the downregulation of survivin is due to inhibition of E2F-1 binding activity to the survivin promoter region.

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